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VOL. 26, SEC. E. AUGUST, 1948 NUMBER 4

FLUORESCENCE MICROSCOPE EXAMINATION OF TRYPANOSOMES IN BLOOD[1]

BY S. STRUGGER[2]

Abstract

With acridinorange it is possible to stain selectively trypanosomes in blood intra-vitam. In subsequent examination by means of a fluorescence microscope only leucocytes and trypanosomes are visible (as shining bright green bodies), while the erythrocytes are not visible. Living and dead trypanosomes, too, may be distinguished in this way.

With the fluorescent dye auramine, trypanosomes in blood may be selectively stained (showing up in golden fluorescence) so that diagnosis for trypanosomes is very greatly facilitated. Even in difficult cases a decision on the presence of trypanosomes in blood is possible.

The staining of blood smears by methods normally used and microscopic examination makes it possible to find trypanosomes in blood easily only if they are present in a sufficient number. But if there are only a few trypanosomes, they may often be overlooked because of the immense mass of deeply colored erythrocytes. In such cases microscopic examination for trypanosomes is usually so difficult that, in practice, it cannot be carried out.

The following paper describes two fluorescence microscope methods with the aid of which microscopic diagnosis for trypanosomes may be so facilitated —even in difficult cases—that even single trypanosomes can be found quickly and accurately. Jancsó (1932 (2)) succeeded in making trypanosomes visible under the fluorescence microscope by the use of trypaflavine. Hirt (1939 (1)) described an intravital staining of trypanosomes in blood of animals that were injected with trypaflavine. Both of these methods have been used mainly for chemotherapeutical purposes.

When Strugger (1940 (3)) discovered that acridinorange (3,6-tetramethyldiaminoacridin) is a vital dye that will stain the protein structures of living protoplasm, he succeeded in finding a method of selective vital staining of living trypanosomes in blood. As fluorochrome a solution of acridinorange 1:10,000—made with 0.85% sodium chloride—is used. A drop of freshly

[1] *Manuscript received January 22, 1948.*

Contribution from the Botanical Institute of the Veterinary College of Hannover, Hannover, Germany.

[2] *At the time, Professor of Botany and Director of the Botanical Institute of the Veterinary College of Hannover. Now, Director of the Botanical Institute and Botanical Garden of the University of Münster i.W. Schlossgarten, Germany.*

taken blood is mixed with a drop of this acridinorange solution on a slide and covered with a cover slip. The examination is carried out with a blue light fluorescence microscope.

Anyone may easily construct such an instrument. The source of light used is a carbon-arc lamp with attached convex lens by which a bundle of parallel rays is produced. The light is filtered by a cuvette (2½ cm. thick) filled with a solution of saturated copper oxide ammonia so that only blue light reaches the plane mirror of the microscope. The microscope is of the ordinary type but has a filter inserted over the ocular. The filter is an orange glass that absorbs the blue light quantitatively but allows green, yellow, and red light to pass through quite unchanged. To focus the fluorescence microscope, a slide, on which is placed pulverized anthracene in liquid paraffin, is used.

With ordinary magnification, the observation of a blood smear stained with acridinorange gives the following image. The erythrocytes are non-fluorescent and scarcely visible. Sporadically situated leucocytes are vitally stained with acridinorange. Their nuclei and protoplasm have a strong green fluorescence. (It may be noted here that a very accurate counting of the leucocytes may be carried out in this way.) The trypanosomes shine with bright, light green fluorescence, do not lose their motility, and are therefore quite easy to find under low magnification. Cytological examination of the living trypanosomes is possible under an oil immersion lens. The cytoplasm fluoresces a diffuse green and the cilia, too, are stained. The nucleus is visible in bright yellow-green fluorescence and the blepharoplast may be very clearly observed.

This method of staining trypanosomes vitally with acridinorange may be used for diagnostic purposes in difficult cases but, because of the different coloration of living and dead protoplasm discovered by Strugger (1940 (3), 1948 (4)), it should be most useful for chemotherapeutical research.

To facilitate microscopic examination for trypanosomes, a simple method has been elaborated that has the same optical advantages as the former and is applicable on dried blood smears.

A smear of blood is made and, after drying, fixed for two to three minutes in methyl alcohol. After a short washing it is stained for four minutes in a solution of auramine (1 part of auramine dissolved in 1000 parts of distilled water, with 5% liquid phenol added after that). Then it is washed in distilled water (one to two minutes) and dried in air. The durability of this stain is satisfactory if the slide is kept in darkness.

Examination of the preparation with the blue light fluorescence microscope is first made at a low magnification (100 to 200×). To find single trypanosomes in difficult diagnostic cases it is well to search first at low magnification and then at higher magnification. The following image is obtained. The background is black. The erythrocytes shine slightly, as dark green circles.

PLATE I

FIG. 1. *Fluorescence microscope photograph of Trypanosoma brucei in blood of a guinea pig, stained with auramine. The erythrocytes are visible as slightly green fluorescent circles. The leucocytes cannot be distinguished. Only the trypanosomes are shining in bright golden fluorescence.*

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The leucocytes cannot be distinguished. Only the trypanosomes are marked by a bright golden fluorescence. Their shape and their inner structure may be excellently observed. The contrast is considerably improved if a drop of liquid paraffin and a cover slip are put on the stained smear.

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THE POTENTIATION OF INSULIN BY SULPHONES[1]

By A. Bruce Macallum[2]

Abstract

Sulphones in trace quantities combined with diet rich in fresh vegetables produce an increased sensitivity to insulin, both in rate of fall of blood sugar levels and maintenance of hypoglycaemia. The sensitivity is not contingent on the presence of sulphone compounds and may persist for several days after these compounds have been detoxicated or eliminated.

The results hereinafter described were obtained from experiments designed to test Keilin's theory that sulphonamide reacted with the zinc in carbonic anhydrase, thus rendering it inactive. If this hypothesis is applied to zinc insulin, it would seem that simultaneous administration of insulin and sulphonamide in rabbits should tend to produce a partial restriction of the hypoglycaemia. This assumption has some apparent support as Greisheimer (3) has shown that sulphonamides produce hyperglycaemia in rats. A preliminary trial with insulin and sulphonamide resulted not in inhibition but in enhancement of the insulin hypoglycaemia. Accordingly the study was reoriented to investigate the potentiating effect of sulphones, as a class, on insulin. This communication is an expanded account of the experiments referred to in an extremely brief preliminary note (7), together with later developments, and is in the nature of an interim report as the problem, which was temporarily suspended owing to wartime controls and postwar teaching load, has been resumed.

Methods

Rabbits were used; 170 single experiments were carried out on 70 animals. Since 1930 the writer has been engaged in studies on insulin synergism and during this period data were accumulated from the behavior of normal animals on insulin in amounts ranging from 0.1 to 0.5 units of insulin per kgm. These are shown in Fig. 2, curve No. 1 and are used as a basis of comparison with insulin in conjunction with sulphones.

The animals were kept in the animal house two to three weeks before use and fed on a standard diet. They were starved 24 hr. before use.

In the first series saturated solutions of sulphanilamide were used. Subsequently, in order to relate the molar concentration of sulphone to the unit value of insulin 1 ml. of a 0.01 *M* sulphone solution was used in conjunction with one unit of insulin. In the case of less soluble preparations more dilute solutions were used but the volume of the dose increased to keep the amount of sulphone in relation to the amount of insulin constant. The sulphone

[1] *Manuscript received in original form July 21, 1947, and, as revised, October 22, 1947. Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont.*

[2] *Research Professor of Biochemistry.*

solutions were injected hypodermically into the side of the animal opposite to the site of insulin administration in order to avoid formation of possible insulin-sulphone complexes.

Blood sugars were determined prior to 1938 by the Folin-Wu method and subsequently by the Shaffer-Hartmann-Somogyi method. All curves of insulin action were plotted using the fasting level as the base and the actual fall from and the return to the initial values expressed as the hypoglycaemia. This eliminated differences in values due to the two methods and makes the results comparable.

The values are expressed in milligram hours. The unit value of insulin is determined by two factors (*a*) the fall in blood sugar values, (*b*) the duration of the hypoglycaemia. A drop of 1 cm. on the ordinate of the graph paper is 10 mgm. % (10 mgm. glucose in 100 cc. blood) and 1 cm. on the abscissa represents one hour, so that 1 cm.^{2} = 10 mgm. hr. Integrating the area of the curve (the shaded areas in Fig. 1) with a metric planimeter and multiplying the total by 10 gives the total mgm. hr. of the hypoglycaemia. These values were used for determining the potentiating effect of sulphones against the same values of insulin alone.

Results

Sulphanilamide and sulphathiazole were the first to be used. These displayed a marked and spectacular increase in sensitivity to insulin. Subsequently the study was extended to include available sulpha drugs and simple and complex sulphones that were readily available. If a sulphone derivative showed no effect on two animals it was rated as negative. Where four out of five animals showed a marked and spectacular increment of the hypoglycaemia they were accepted as positive.

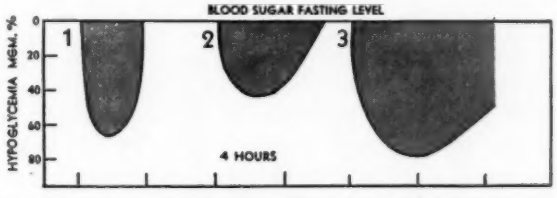


FIG. 1. *Curve No. 1: 0.1 units insulin, 1.5 ml. 0.01 M taurine solution per kgm.; No. 2: standard composite curve, 1.5 units insulin per kgm.; No. 3: 1.5 units insulin, 0.75 ml. 0.01 M saccharin per kgm.*

Typical examples of potentiation are shown in Fig. 1. Curve No. 2 is a composite, the data being obtained from a series of normal insulinized rabbits on 1.5 units per kgm. The points on the curve represent the arithmetical mean of decreases in blood sugar values. Curve No. 3 is the potentiating effect of 0.75 ml. 0.01 *M* saccharin on 1.5 units insulin per kgm. This experiment was terminated after eight hours, before the blood sugar had returned to the fasting level. Curve No. 1 is the effect of 1.5 ml. 0.01 *M*

taurine solution on 0.1 units insulin per kgm. Here the insulin effect has been increased approximately fourfold. Where the standard ratio of sulphone/insulin failed to give a complete result in the eight hour period it was found necessary to reduce either the amount of sulphone or insulin. This was employed when the effects were catastrophic, accompanied by a high mortality rate from convulsions when the standard ratios were used.

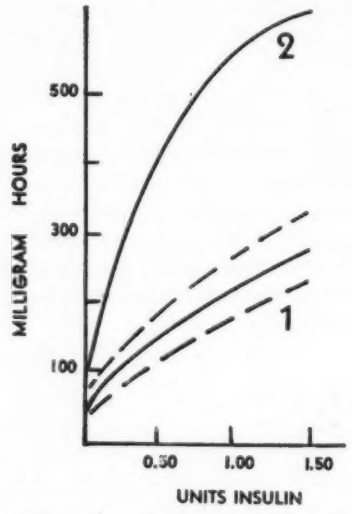


FIG. 2. *Curve No. 1: standard normal curve of insulin—dotted lines indicate standard deviations from mean; No. 2: potentiating effect of 1.5 ml. 0.01 M taurine per kgm.*

Fig. 2 illustrates the sensitizing effect of taurine (1.5 ml. of 0.01 *M* per kgm.) on varying doses of insulin. In the lower curve (No. 1), the solid line represents the effect of varying amounts of insulin in rabbits (the values on the curve being statistically corrected), the dotted lines indicating the standard deviations. The upper solid line (No. 2) demonstrates the effect of the sensitizing action of taurine on the insulin. The 1.5 unit value in this case was calculated from an incomplete experiment described previously, the blood sugar levels not returning to the base line in eight hours. Projecting the curve back to the base line in this case gives a value that, if used, would make the curve register a much higher value for 1.5 units of insulin.

In all cases where definite increases in sensitivity to insulin were brought about by sulphones, the effect on insulin alone, subsequently administered, persisted in a gradually decreasing degree from 3 to 15 days, occasionally longer, after termination of sulphone administration.

A summary of the relative effects of the various members of the sulphone class investigated is shown in Table I where they are classified in their main chemical groupings. In the case of simple sulphones the potentiation did

not appear until sulphamide was used and the activity increased in the subsequent members of this group, the maximum effect being attained with phthalyl tauramide. Tauramide hydrochloride (not shown in the table) was also tested and was about equal to taurine. In the case of sulpha drugs the potency was least with sulphanilamide, but increased in the succeeding members of this series, the last, No. 307, a disulphone under experimental trial, being the most effective in this group. In the benzene sulphonic derivatives, benzene sulphonic acid was questionable but the other members of this group were the most active of all of the sulphones investigated.

These observations were completed at the end of 1942, during a period when the animals were supplied from a single dealer who raised them on a specified diet for some weeks prior to delivery. Subsequently this source of supply ceased and the extension of wartime controls and the increased cost of feed made it necessary to use animals available regardless of source or dietary history and a commercial chow diet was substituted for that previously used. The commercial diet apparently did not differ from the previous standard diet in regard to its effect on sugar tolerance, behavior of the animal to insulin, and maintenance of general nutritional levels. However, animals on this regime displayed reduced potentiation when sulphones were used in conjunction with insulin and only sulphones that gave the maximum effect showed even moderate activity.

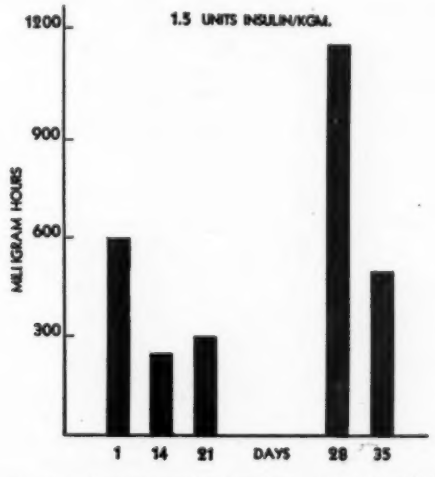


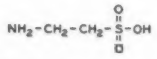
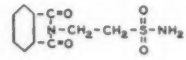
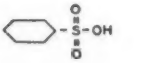
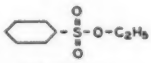
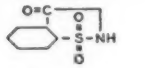
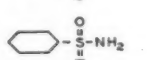
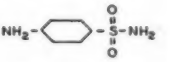
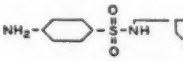
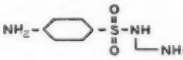
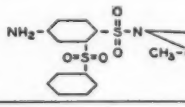
FIG. 3. *All animals get 1.5 units insulin per kgm. Day 1, added 1.5 ml. 0.01 M taurine per kgm. Days 1 to 21, commercial diet. Days 22 to 35, standard laboratory diet.*

Fig. 3 illustrates this point and was typical of every animal in this group on the commercial diet. Insulin was given periodically on the days indicated in the diagram. On the first day 1.5 ml. of 0.01 *M* taurine was given with the insulin, but was discontinued thereafter. For the first 21 days the animals

were on the commercial diet but on the 22nd day the standard diet used in the earlier series of experiments was used. The taurine effect on the first day was not so pronounced and the insulin effect on the 14th and 21st day was within the normal range. After seven days on the standard diet (the 28th

TABLE I

DEGREE OF POTENTIATION OF INSULIN ACTION BY VARIOUS SULPHONES

	Formula	Degree of potentiation
Simple sulphones		
Sulphamic acid		–
Ammonium sulphamate		?
Sulphamide		+ +
Taurine		+ + +
Phthalyl tauramide		+ + +
Benzene sulphonic acid derivatives		
Benzene sulphonic acid		?
Ethyl benzene sulphonate		+ + + +
Saccharin		+ + + +
Benzene sulphonamide		+ + + +
Sulphonamide drugs		
Sulphanilamide		+ +
Sulphathiazole		+ + +
Sulphaguanidine		+ + +
Sulphadiazine		+ + + +
Expt. No. 307		+ + + +

day) insulin was given with most spectacular results, which persisted even on the 35th day or longer. These had never occurred previously in many years of observation on insulinized animals. This experiment illustrates the persistence of the sensitizing effect of the sulphones, which was the result of the conjoint action of the sulphone and an unsuspected trace factor in the diets used prior to the change to the commercial diets. This factor was previously unsuspected and not manifested till the change in feeding took place. Every animal in this series displayed this latent effect of the sulphone and the dietary factor, some even more spectacularly than that shown in the illustration. The standard diet contained fresh cabbage, lettuce, carrots, hay, and oats—fresh vegetables being absent from the commercial diet, which was made up from mixtures of mill feeds. It is therefore apparent that the potentiating effect shown in Table I is due to the combined effect of the sulphone sensitization and some element in the fresh vegetables in the standard diet.

The crossover test usually applied in insulin standardization cannot be used without considerable modification in conjunction with sulphones. Owing to the persistence of the potentiating effect, crossing in 24 hr. intervals is out of consideration. At least two weeks must elapse before the normal insulin levels of action return and even then not all animals may be free of sulphone effect. Again a milder preparation must be used to avoid possible death from hypoglycaemic shock so that these conditions tend to render the crossover test less certain. Table II illustrates a modified crossover test, which, under the conditions referred to above, is about the best that can be expected

TABLE II

EXTENT OF HYPOGLYCAEMIA IN MILLIGRAM HOURS IN MODIFIED CROSSOVER TEST

	Group A	Group B
Day 1	1.5 Units insulin per kgm. 1.5 Ml. 0.01 *M* tauramide per kgm.	1.5 Units insulin per kgm.
	650 Mgm. hr.	420 Mgm. hr.
Day 14	1.5 Units insulin per kgm.	1.5 Units insulin per kgm. 1.5 Ml. 0.01 *M* tauramide per kgm.
	440 Mgm. hr.	**560** Mgm. hr.

in view of the 14 day interval and the predictable activity of a sulphone of moderate sensitivity. While smaller amounts of insulin might have been used, subconvulsive levels of blood sugars were desirable to approximate more closely the actual testing conditions for determining unit values.

Discussion

The possibility that insulin forms a slowly diffusible complex with sulphones has been advanced and Sahyun and Heyn (6) found that insulin and brom-

camphor sulphonic acid, camphoric and camphoronic acids, when mixed under specified conditions, formed slowly acting depots with prolonged hypoglycaemic effect. The writer noted that sulphones and insulin mixtures in several cases formed precipitates or colloidal suspensions. To avoid the possibility of 'depots', insulin and sulphones were administered separately on opposite sides of the test animal. The writer's findings do not support the above view. The fall in blood sugar levels is extremely rapid and catastrophic and the sulphanilamide appears in the blood plasma in two hours and disappears about an hour afterwards, long before the blood sugar levels return to the initial fasting levels. Furthermore the sensitivity to insulin once established persists for days or weeks. Again the presence of a dietary factor in the potentiating mechanism together with these facts indicate that much more is involved than an insulin 'depot' and the effect is comparable to the action of synergists of pancreatic and duodenal origin (1, 4, 5).

Sulpha drugs in therapeutic doses do not depress blood sugar levels. Greisheimer *et al.* (3) found that in the range of 100 mgm. per kgm. the effect was hyperglycaemic. In the present investigation doses were of the order of 2 to 5 mgm. per kgm. Goldberg and Jefferies (2) used comparable doses in investigating the potentiating effect of sulphanilacetic acid. Some of the sulphones in these trace quantities showed slight hypoglycaemic action when used alone, but those most active in activating insulin had no effect on the blood sugar levels.

At this stage no discussion of the sensitizing mechanism is included owing to the presence of the uncontrolled variable—the trace factor in the green vegetables of the standard diet—whose activity was manifested in experiments of which the example in Fig. 3 is typical. Further investigations as to the nature and action of this component are in project.

Acknowledgments

The writer wishes to express his indebtedness to Dr. Christian Sivertz, who was associated with the earlier phases of the investigation, for his continued interest and helpful suggestions. Also to Dr. Oliver Kamm of Parke-Davis Co. for supplies of phthalyl tauramide and No. 307, to Dr. R. O. Roblin, Jr., American Cyanamide Co., who furnished the tauramide and tauramide hydrochloride.

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THE FORMATION OF ACETATE IN BRAIN TISSUE SUSPENSIONS[1]

By J. L. Webb[2] and K. A. C. Elliott[3]

Abstract

Observations have been made on methods for the determination of acetate and acyl phosphate in tissue suspensions. Previous work on the formation of acetate by respiring brain suspensions, especially in the presence of added pyruvate, has been confirmed. No evidence could be obtained that the substance formed is actually acetyl phosphate, which breaks down to yield acetic acid in the course of the estimation or enzymatically during the incubation of the tissue. The amount of acetate formed in brain suspensions is increased by the presence of malonate. It seems also to be increased by the addition of malate or α-keto-glutarate and by thiamine. Spontaneous breakdown of pyruvate could account for part of the acetate formed from pyruvate added to tissue. Fluoroacetic acid interferes with the determination of acetic acid. There was no evidence that it increases the formation of acetate by rat brain tissue suspensions. It does not affect the oxygen uptake of these suspensions with glucose or pyruvate as substrates.

Much work in recent years has indicated that some reactive two-carbon compound or radical is a key intermediate in the metabolism of carbohydrate and of fat and related substances via the Krebs cycle and in the synthesis of certain complex molecules. Such a two-carbon body is probably also the source of the acetyl group of acetylcholine, which appears to be an essential substance in the functional chemistry of nervous tissue.

Acetic acid is well known to be produced from alcohol, pyruvate, and other substances by various microorganisms. Among animal tissues, Krebs and Johnson (12) and Elliott *et al.* (6) found that, anaerobically, testis caused a dismutation of pyruvate whereby one molecule of pyruvate was reduced to lactate while another molecule of pyruvate was oxidized to acetate and carbon dioxide. The dismutation occurred to a considerably smaller extent in tissues other than testis.

Weil-Malherbe (22, 23) detected the formation of small amounts of acetate from pyruvate by minced ox brain, both anaerobically and aerobically. Aerobically, Long (16) found acetate production from pyruvate with pigeon brain mince and estimated that about 25% of the pyruvate used was converted to acetate. With rat brain suspensions, Elliott *et al.* (8), using accurate analytical procedures, found that about 14% of the pyruvate utilized aerobically was oxidized to acetate. These authors also found that small amounts of acetate were produced during the oxidative metabolism of glucose and lactate.

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Contribution from the Department of Neurology and Neurosurgery, McGill University, and the Montreal Neurological Institute, Montreal, Que. This work was aided by a grant from the National Research Council of Canada.

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Acetate is consumed by intact animals (see for example (3, 20)) and by various tissues *in vitro* (4, 6, 24). However, Elliott *et al.* (6, 8) found no sign of any utilization of acetate by rat brain tissue slices or suspensions. It seemed possible, therefore, that acetate is not a normal product of brain metabolism. Possibly a different substance that estimates as acetate in the analytical method is the actual metabolite formed. Such a substance might be acetyl phosphate, which is readily hydrolyzed to acetic acid and phosphate and which is an intermediate in the oxidation of pyruvate by certain bacteria (14). Or acetate formation may be an abnormal process occurring under *in vitro* conditions. In an interesting study of the metabolism of the malaria parasite, Speck *et al.* (21) found that acetate formation from pyruvate was greater with free parasites than in parasitized erythrocytes, possibly because the erythrocyte provides a more 'normal' environment, and was increased by high pyruvate concentrations. Additions of catalytic amounts of the dicarboxylic acids of the Krebs cycle slightly decreased the acetate production by free parasites, while malonate, which interferes with the cycle, increased the acetate formation. It was suggested that pyruvate, or a reactive two-carbon fragment formed from pyruvate, is normally taken up and oxidized via the Krebs cycle but under some conditions it may be transformed into acetate.

In the present study, isotonic brain suspensions, which have been shown to be comparable to brain slices in rate and type of metabolism (7) and lend themselves to accurate analytical studies, have been used. The observations of Elliott *et al.* (8) on acetate formation have been confirmed. No evidence that the substance actually formed is acetyl phosphate could be obtained. Acetate production was possibly slightly increased when the tissue was cytolyzed by homogenization in hypotonic medium, a condition that might perhaps be compared with that of the free malaria parasites mentioned above. Acetate formation was considerably increased in the presence of malonate but it was also somewhat increased when malate or α-keto-glutarate was added. An appreciable amount of acetate is formed from pyruvate under the experimental conditions in the absence of any tissue.

Bartlett and Barron (1) and Kalnitsky and Barron (11) have found that fluoroacetate inhibits the oxidation of acetate by kidney, liver, and muscle and by yeast and certain bacteria, and that in the presence of fluoroacetate the utilization of added pyruvate is diminished while acetate accumulates. They concluded that oxidation of pyruvate to acetate is of great importance in animal tissues. We have obtained no evidence that fluoroacetate interferes with the respiration of rat brain suspensions with either glucose or pyruvate as substrate, or that it increases the accumulation of acetate very markedly. Fluoroacetate interferes considerably with the determination of acetic acid.

In preparation for this work some observations have been made on the methods for acetate and acetyl phosphate determinations.

Methods

Suspensions, containing usually 150 mgm. tissue per ml., were prepared from whole rat brains by homogenization in calcium-free Ringer–0.017 *M* phosphate solution by means of the apparatus of Potter and Elvehjem (17). In some cases 0.11 *M* sodium phosphate, pH 7.3 to 7.5, was substituted for sodium chloride in the medium. Samples of the suspension, usually 3 ml., were pipetted into Barcroft manometer flasks and incubated for an hour with shaking at 38° C. Substrates, inhibitors, etc., or equivalent amounts of sodium chloride, dissolved in 0.3 ml. water, were introduced into the flasks before the suspension. The gas phase in the flasks was air. When oxygen uptake was to be measured, the center wells contained alkali-soaked filter paper rolls. At the end of the experimental period, the suspension from two flasks was pooled and samples were taken for acetic acid or acetyl phosphate determinations. A number of determinations were also made on suspensions deproteinized immediately after homogenization.

Acetic acid was determined by the method of Elliott *et al.* (8). The suspension was deproteinized with zinc sulphate and sodium hydroxide, and a 5 ml. sample of the filtrate, representing 1.7 ml. of tissue suspension or 250 mgm. of tissue, was run into the distillation flask, which contained 2 gm. of acid potassium phosphate. Steam distillation was carried on at atmospheric pressure until 50 ml. of distillate had collected. The distillate was rendered alkaline, evaporated down, and redistilled with 0.5 ml. of phosphoric acid. The second distillate was aerated with carbon dioxide-free air and titrated with freshly standardized carbon dioxide-free 0.005 to 0.008 *N* sodium hydroxide using bromthymol blue as indicator. The double distillation almost completely obviates interference by lactic and pyruvic acids. The apparatus at first used was copied, evidently imperfectly, from apparatus that was lent to one of us (K.A.C.E.) by Dr. W. C. Stadie and used in earlier work. It was found that variable and often very high blank titrations were obtained owing to traces of phosphate being carried into the distillate. This disturbance was mainly due to the collection of spray and condensed steam in the neck of the flask (*A* in Fig. 1) where it partially blocked the neck so that rapidly flowing steam could carry droplets of this collection further and into the condenser. Satisfactory results were obtained when new apparatus was constructed in which the neck was widened, the tube (*B*) to the Kjeldahl trap was widened, and a perforated bulb (*C*) was used for the steam inlet to allow smoother distillation. In Table I results of tests of the method are shown.

The method determines acid volatile with steam under the conditions used and is not specific for acetic acid. Acetate is the most probable substance that is formed in the tissue and fulfils these conditions but it is recognized that throughout this study the term "substance estimated as acetate" should be used instead of "acetate." All determinations were done in duplicate. But differences in individual figures in Tables II, IV, and V up to 2 μmoles per gram are within the limits of errors of the method and are probably not significant.

TABLE I

ACETIC ACID DETERMINATION. BLANKS AND RECOVERIES

The 'blanks' and 'samples' consisted of 5 ml. of solution containing 2 mgm. of sodium pyruvate and 2 mgm. *dl*-lactic acid and the amount of acetic acid shown

	Titration, ml. 0.005 *N* NaOH, average (range)	Equivalent acetic acid, mgm., average (range)	Recovery* %
Distilled water	0.16 (0.09-0.20)	0.05 (0.03-0.06)	
Complete distillation blanks	0.24 (0.15-0.38)	0.07 (0.05-0.11)	
Sample 0.27 mgm. acetic acid	1.15	0.34	100
" 0.35 " " "	1.38	0.41	97
" 0.47 " " "	1.80 (1.76-1.86)	0.54 (0.53-0.56)	98-102
" 0.71 " " "	2.58	0.77	98

* *Corrected for average distillation blank.*

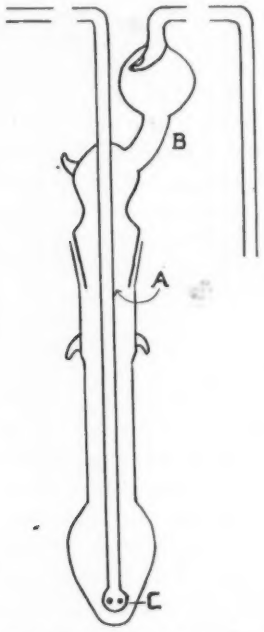

FIG. 1. *Steam distillation flask for acetic acid determination. The total height is 25 cm. The clearance at point A is at least 5 mm.*

Acetyl phosphate was determined by adaptations of the method of Lipmann and Tuttle (15), which consists in treating the sample with freshly neutralized excess of hydroxylamine hydrochloride solution, pH 6.4. The hydroxylamine reacts with acyl phosphate to give the hydroxamic acid. After deproteinization, the filtrate is treated with ferric chloride, which produces a brown-purple

color with the hydroxamic acid formed. The depth of color is measured with a photoelectric photometer. The color is compared with standards prepared from succinic anhydride, which gives the monohydroxamic acid. The color developed with ferric chloride in the standard corresponds to 80% of that given by an equivalent amount of the acetohydroxamic acid formed from acetyl phosphate.

In the original method, deproteinization was achieved with trichloracetic acid. With the tissue suspensions used here, it was found that cloudy filtrates were always obtained after the addition of even very high concentrations of trichloracetic acid, when high concentrations of hydroxylamine were present.* Perchloric acid was similarly unsatisfactory. Metaphosphoric acid gave clear filtrates but prevented the development of color with ferric chloride. In an alternative procedure described by Lipmann and Tuttle for use when there were present high concentrations of phosphate that depress the intensity of the color developed, a solution of zinc chloride and calcium chloride was added, followed by an amount of sodium hydroxide approximately equivalent to the zinc. However, we found that the strong buffering action of the hydroxylamine prevented the necessary formation of zinc hydroxide so that deproteinization was not achieved. In the method adopted here, sufficient relatively strong sodium hydroxide was added to overcome the buffer action of the hydroxylamine. This produced a copious precipitate, with deproteinization, and a clear filtrate was obtained. However, in spite of the presence of more calcium than was theoretically sufficient to precipitate the phosphate, lower colorimeter readings were always obtained when the original medium contained high concentrations of phosphate. For these experiments, therefore, the standard solutions always contained phosphate concentrations equal to those in the unknowns. In view of the high buffering action of hydroxylamine, the addition by Lipmann and Tuttle of a small amount of acetate buffer to the reaction mixture was pointless and was omitted here. The method finally adopted was as follows.

Two ml. of tissue suspension was added to 2 ml. of 2*M* hydroxylamine, adjusted to pH 6.5 to 6.6, in a 15 ml. centrifuge tube and the mixture was allowed to stand for 10 min. with occasional stirring. Then 1 ml. of 0.6*N* sodium hydroxide was added to bring the pH to about 7.0, 2 ml. of 3.4% zinc chloride – 5.6% calcium chloride solution was added with stirring and finally 2 ml. more of 0.6*N* sodium hydroxide was added slowly with stirring. After centrifuging, 4 or 5 ml. of the clear supernatant fluid was treated with 1 ml. each of 3*N* hydrochloric acid and 5% ferric chloride in 0.1*N* hydrochloric acid. After standing for 5 to 30 min. the color was determined with the Klett-Summerson colorimeter with filter No. 540. Recoveries of succinhydroxamic acid standard solutions added to brain tissue suspensions in either Ringer-phosphate or 0.11*M* phosphate ranged from 94 to 106%.

* *Dr. Lipmann has since informed us that a considerably lower concentration of hydroxylamine may be used.*

Results

In Table II results of acetic acid determinations before and after incubation of isotonic suspensions are shown. The amounts of acetate formed varied but the average in the presence of added pyruvate is in accord with the amount formed after two hours' incubation, 9.9 μmoles per gram, calculated from the results of Elliott *et al.* (8). Without added pyruvate, smaller

TABLE II

PRODUCTION OF ACETIC ACID IN ISOTONIC RAT BRAIN SUSPENSIONS

Micromoles of acetic acid per gram of whole brain; incubation one hour at 38° C.

Substrate	Initial	Incubated	Increase	Substrate	Incubated	Increase* (average)
Pyruvate, 0.025 *M*	—	10.6	—	Pyruvate,	11.5, 9.2, 8.9	8.3
	0.6	7.5	6.9	0.25 *M* in		
	0.5	6.3	5.8	0.11 *M*		
	0.5	9.7	9.2	phosphate		
	3.9	8.7	4.8			
	1.8	5.4	3.6	Glucose, 0.01 *M*	2.2, 6.3, 5.3, 5.0	3.2
	2.0	6.8	4.6			
	0.5, 0.7, 2.2, 3.2	—				
Average	1.6	7.8	6.2	No addition	6.2, 4.4, 3.8, 5.0	3.2
No addition (insulinized)	0.6	1.9	1.3			

* *After deducting average initial figure.*

amounts of acetate are formed. This is probably produced from the traces of pyruvate that are present in the respiring tissue as an intermediate in glucose or lactate oxidation. Fresh brain suspensions, even without added substrate, always contain appreciable amounts of lactate formed from residual blood glucose. The amount of lactate, and hence of acetate formed, is much reduced in brain from insulinized rats (8, 10).

Acyl Phosphate

Results of acyl phosphate determinations are shown in Table III. The amounts of acyl phosphate present were very small and often below the range of accuracy of the determination. The amount of acyl phosphate found did not increase with time of incubation but rather decreased and was considerably less than the equivalent of the acetic acid usually found. It is evident therefore that the substance estimated as acetic acid is not produced from acetyl phosphate by hydrolysis in the course of the acetic acid determination. Lipmann (13) has found a very active acyl phosphatase in many tissues that splits acetyl phosphate to acetic acid and phosphate. Experiments with the brain suspensions showed that about 70% of added lithium acetyl phosphate,*

* *Kindly given to us by Dr. Fritz Lipmann.*

TABLE III

ACYL PHOSPHATE IN RAT BRAIN SUSPENSIONS

Micromoles of acyl phosphate* per gram

Substrate	No. of expts., averaged	Incubation period		
		Initial	30 min.	60 min.
Pyruvate, 0.025 *M*	3	2.3	1.4	1.3
Glucose, 0.01 *M*	5	3.2	3.1	2.3
No addition	3	1.7	1.4	1.2
Pyruvate, 0.025 *M* in 0.11 *M* phosphate medium	3	1.5	1.1	1.1

** Figures are only approximate since a number of results fell below 1.5, which is about the lower limit of reliability of the method.*

0.007 *M*, was split in five minutes at 38° C. It therefore seemed possible that acetyl phosphate might be formed by the tissue but be hydrolyzed during the incubation period. Lipmann (13) and Shapiro and Wertheimer (19) have shown that acyl phosphatase is strongly inhibited by high concentrations of phosphate. This was confirmed with brain suspensions in isotonic, 0.11 *M*, phosphate in which the rate of destruction of added acetyl phosphate was decreased by about 85%. Brain suspensions in isotonic phosphate with added pyruvate produced acetic acid at least as fast as in Ringer-phosphate medium (see Table II) but again no sign of increased acyl phosphate was found. It thus seems unlikely that acetyl phosphate is an intermediate in the production of acetate by brain tissue. Small amounts of a substance that is estimated as acyl phosphate, about 2.4 μmoles per gram (average of seven determinations), were found even when the tissue was homogenized directly in medium containing 2*M* hydroxylamine to trap acyl phosphate already present in the tissue. It is possible that the substance estimated in this case is phosphoglyceryl phosphate, which also forms a hydroxamic acid yielding color with ferric salt, and which might be present in traces in tissue in which glycolysis is proceeding or has recently occurred. The amounts are so small and erratic, however, that they may represent nonspecific interference with the method by tissue materials.

The oxygen uptake rate of isotonic brain suspensions even in the presence of 0.11*M* phosphate was not affected by the addition of lithium acetyl phosphate, 0.007*M*.

Effects of Various Additions on Acetate Formation

In Table IV the effects of various factors on acetate formation are shown. Malonate addition increased the acetate production from pyruvate definitely and considerably. Malonate had only a faint inhibitory effect, about 5%, on the oxygen uptake rate of isotonic suspensions in the presence of added pyruvate or glucose. Addition of small amounts of malate or α-keto-glutarate

TABLE IV

EFFECT OF VARIOUS FACTORS ON ACETATE PRODUCTION

Micromoles of acetic acid per gram; incubation one hour at 38° C.

Isotonic suspensions		Hypotonic suspensions	
Additions	Acetate formed*	Additions	Acetate formed*
Pyruvate, 0.025 *M*	6.2 (average**)	Pyruvate, 0.025 *M*	9.0, 6.5, 7.5, 14.2
Pyruvate and malonate, 0.02 *M*	12.1, 11.0, 16.7, 14.6, 13.4	Glucose, 0.01 *M*	3.1, 9.6, 5.0
Pyruvate and malate, 0.005 *M*	5.5, 10.7, 9.1, 11.8	Pyruvate and malonate, 0.02 *M*	13.2, 13.9
Pyruvate and α-keto-glutarate, 0.005 *M*	10.6, 12.3	Pyruvate and malate, 0.005 *M*	7.5, 8.1
Pyruvate and thiamine, 0.003 *M*	6.5, 11.9, 9.4, 10.1	Pyruvate and malate and malonate	9.5, 10.6

* *After deducting average initial figure.*
** *From Table II.*

also seemed to increase the acetate production of isotonic suspensions slightly. (In previous work on similar suspensions (7) it was found that malate and fumarate produced an initial small increase in the oxygen uptake rate.) The addition of thiamine also seemed to increase acetate formation. Omission of magnesium ion from the medium or a fivefold increase in magnesium concentration had no obvious effects. With tissue in which the respiratory system was disrupted, by homogenization in hypotonic medium (7), the average amount of acetate produced was slightly increased but statistically the increase was of doubtful significance.

The addition of 0.02 to 0.1*M* fluoroacetate* caused no appreciable inhibition of the oxygen uptake of rat brain suspensions in the presence of either glucose or pyruvate. (Bartlett and Barron (1) found slight inhibition of respiration of rabbit brain slices but considerable, 53%, inhibition with guinea pig brain slices, which, they state, can oxidize acetic acid.) The acetate determination showed the presence of large variable amounts of steam-volatile acid. However, it was found that fluoroacetic acid is appreciably volatile with steam and causes large and variable blank titrations when it is present in the solution distilled. Three pairs of acetate determinations on tissue suspensions incubated with pyruvate and 0.02*M* fluoroacetate gave total 'acetate' values varying from 11.3 to 29.5 μmoles, averaging 19.7 μmoles per gram. Three pairs of blank determinations in which the pyruvate and fluoroacetate were incubated in the absence of tissue gave values varying from 8.4 to 18.6, averaging 10.9 μmoles, in the amount of solution that would contain 1 gm. of tissue. The amount of acetate formed with tissue present might thus be about 8.8 μmoles, the difference between these averages.

* *We are grateful to Dr. E. S. G. Barron for the gift of a sample of sodium monofluoroacetate.*

This amount is not very different from that formed with pyruvate and tissue alone and we cannot conclude from these results that fluoroacetate has a significant effect on acetate formation in rat brain tissue suspensions. Similar results were obtained with glucose as substrate and with 0.1*M* fluoroacetate with glucose or pyruvate.

Nonenzymic Formation of Acetate

Pyruvic acid is a highly reactive substance. Among other transformations that it has been reported to undergo is a decomposition to acetic acid on warming to 50° C. in dilute sodium carbonate solution (9). It was found that even in the extremely mild conditions here used, one hour at pH about 7.4 and 38° C., a slight amount of volatile acid is produced in the absence of tissue (see Table V).

TABLE V

NONENZYMIC FORMATION OF ACETATE FROM PYRUVATE

Incubated for one hour at 38° C., pH 7.2 to 7.4

Medium	Pyruvate added, μmoles/ml.	Ascorbate added, μmoles/ml.	Acetate formed	
			μmoles/ml.	Equivalent μmoles per gm. tissue, average
Ringer-phosphate	25	—	0.70, 0.36, 0.23, 0.05	2.2
"	25	15	3.4, 4.1	25.1
Tissue suspension	25	15	3.6, 2.7	20.7
Redistilled water	25	15	2.8, 6.0	29.1
Ringer-phosphate and 0.02 *M* malonate	25	15	4.9	29.8
Ringer-phosphate	12	30	7.9	47.6
"	25	0.3	0.2, 0.4, 0.6	2.7
"	1	—	0.3, 0.18, 0.12	1.0
"	1	0.3	0.11, 0.12, 0.25, 0.63	1.9

Cavallini (5) reported that, in the presence of ascorbic acid, pyruvate is oxidized to acetate and carbon dioxide in the acid to neutral pH range. In the abstract of Cavallini's work available to us, there is no suggestion of the mechanism of this reaction. Ascorbate is readily oxidized especially in the presence of traces of copper ion. Hydrogen peroxide, which is formed in the oxidation of ascorbate, would oxidize pyruvate to acetate and carbon dioxide. Alternatively, dehydroascorbate might react with the pyruvate. Results shown in Table IV confirm the report of Cavallini. Relatively high concentrations of pyruvate, 0.025*M*, and ascorbate, 0.015*M*, incubated together in neutral aqueous or Ringer-phosphate solution, yielded considerable amounts of a substance estimated as acetate. This occurred in the presence or absence

of tissue and in experiments in which the reactants were dissolved in water that had been twice redistilled in glass apparatus to minimize contamination with copper. Rat brain contains about 0.36 mgm. ascorbic acid per gram (2). A suspension containing 150 mgm. brain tissue per ml. might therefore contain $0.0003M$ ascorbate. Such a low concentration was not found appreciably to affect the amount of acetate formed from pyruvate in the absence of tissue.

Discussion

It thus appears that spontaneous breakdown and perhaps, to a slight extent, the reaction with ascorbic acid, could account for a considerable fraction of the acetate production in tissue suspensions. When metabolic processes are disrupted by the addition of malonate or by homogenization in hypotonic medium the accumulation of acetate is usually increased. This suggests the possibility that formation of free acetic acid by brain tissue is not a normal physiological process but may result from abnormal conditions existing *in vitro*. The results with fluoroacetate, an inhibitor of acetate oxidation in various organisms, do not suggest that acetic acid is formed and then further oxidized to any considerable extent. This agrees with previous results showing that added acetate is not metabolized by rat brain (6).

The possibly significant increases in acetate formation in the presence of malate and α-keto-glutarate suggest that these give rise to an increased concentration of an intermediate from which acetate may be formed. The apparent effect of added thiamine suggests that it may be concerned in the production of this intermediate or in a process that can produce acetate. However, it is probable that work with thoroughly disintegrated tissue or with tissue extracts, in which the respiratory systems are disrupted and the effects of various additions are more marked (see for example, Reiner (18)) would be necessary to clarify the mechanism of acetate production.

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THE INGESTION OF SEA WATER AS A MEANS OF ATTENUATING FRESH WATER RATIONS[1]

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Abstract

Though the drinking of undiluted sea water by the castaway has been shown to be dangerous, there have been few studies on the use of sea water diluted with fresh water. The experiment was designed to permit a statistical evaluation of relative gain or relative loss in body weights to human subjects, from drinking a daily supplement of sea water. The addition of 10 oz. of sea water to the daily ration of 16 oz. of fresh water resulted in a mean (corrected) weight saving of 2.24 lb. over a period of six days. This result was significant. That this relative gain in weight was beneficial is supported by the subjects' unanimous opinion to this effect, by the specific gravities of blood and urine, and by the figures for blood and urine electrolytes in control and experimental subjects.

Introduction

The purpose of this investigation was to determine the possible value of drinking sea water with fresh water rations, as a means of retarding dehydration. The experiment was so designed that a statistical evaluation of the changes in body weight in the treated and control groups was possible (Fisher (5)).

The drinking of undiluted sea water by the castaway has been shown, beyond reasonable doubt, to be a dangerous procedure. The records of survivors from sea disasters and data obtained in the laboratory are in agreement. Elkinton and Taffel from their studies on dogs (3) and Winkler and his co-workers from studies on humans (11) concluded that the intracellular dehydration accompanying the drinking of sea water was progressive, though the extracellular fluid tended to be maintained. A similar conclusion was reached by Gamble (8),—that though salt (sea) water will assist in the removal of solutes (including nitrogenous waste products) it does so at the expense of intracellular fluid. Elkinton and Winkler (4) in reviewing their work on the effects of drinking undiluted sea water, felt that no value could be expected from the use of diluted sea water.

Rectal administration of sea water had been suggested as a means of combating dehydration, the theory being that the colon is capable of selective absorption of water from its contents. It was shown (1, 7), however, that the colon did not concentrate sea water, and that this method was inadvisable as dehydration was furthered by the procedure.

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Contribution from No. 1 Clinical Investigation Unit (R.C.A.F.), Toronto, Ont., the Department of Pharmacology, Dalhousie University, Halifax, N.S., and the Department of Biology, University of New Brunswick, Fredericton, N.B. This work, carried out as a wartime project (Nov. 1944–March 1945), was supported by a grant from the Associate Committee on Aviation Medical Research, National Research Council, Ottawa. Presented in part before the Nova Scotian Institute of Science, February 1947.

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A frequent objection advanced against drinking sea water is that the strongest solution of sodium chloride the kidneys can excrete is slightly less than 2% (2), whereas sea water contains approximately 3% of sodium chloride.

The arguments in favor of drinking sea water relate to the fact that men on short water rations are in chloride debt as the result of the continued loss of chloride in the urine and sweat. There is also a loss of sodium with the chloride, and the depletion of the extracellular fluid volume that results may be the important determining factor in survival time.

In an evaluation of the experience of five survivors who had been adrift for 15 days, Good (9) suggests that "adding small amounts of salt water to the daily ration of fresh water so that the potability of the water is not changed, seems to be an excellent idea as it will make the water last longer, will tend to keep the blood chlorides at a normal level, and should have a tendency to satiate thirst." The five survivors he studied had added a small amount (actual amount not specified) of sea water to their daily ration of fresh water for 13 days. For the last two days there was no fresh water. Two of the survivors then drank undiluted sea water and subsequently wanted to cut their own wrists.

Ladell's studies on humans (10) indicate that there is a slight but definite relative gain in body weight from drinking diluted sea water. He also pointed out that 400 cc. of sea water daily is the maximum amount that can be taken into the body, without calling on the body's reserves to excrete or to retain salt. However, the British Medical Research Council in taking account of factors not specified, advised against the drinking of sea water, although soaking biscuits in it was permitted. The United States Army Air Forces' manual *Survival* recommends the mixture of one part of sea water in six parts of fresh water, as a means only of replacing salt lost by sweating during hot weather.

A Russian naval officer who survived 36 days in a small boat in the Black Sea reports (12) that he and three other men with him drank sea water along with their small ration of fresh water and what they could obtain from rain. Though he was the only man who survived (the others died on the 19th, 24th, and 30th days) he states that they became accustomed to drinking the sea water, and that two years later he finds that fresh water and ordinary food still taste undersalted.

Material

Human volunteer subjects were used in all experiments herein reported. A number of preliminary studies were performed that showed that no more than an equal volume of sea water with fresh water could be tolerated for more than one day, and that even this concentration was apt to cause nausea. As a result of these studies a proportion of 10 oz. of sea water to 16 oz. of fresh water per day was set for subsequent experiments. The two controlled experiments were conducted with six airmen (three in each experiment) together with one medical or medical associate officer acting both as subject

and 'guide' during each experiment, giving a total of 48 test days. Another earlier test, involving four men for six days, was performed, but owing to a difference in the arrangement of the test procedure, it was not included in the final analysis. All the evidence secured from the results of this test pointed to conclusions similar to those obtained from the two tests herewith reported. The selection of subjects was made after a thorough physical examination and history-taking. Those passing the examination were then selected by alphabetical priority.

All experiments were performed in a 'tropical room.' Toilet facilities were provided in the chamber. During the day, from 9 a.m. to 6 p.m. the temperature was kept at 30° C., with water vapor pressure at 20 mm. Hg. At night the dry bulb temperature was 20° C. and water vapor pressure was 13 mm. Hg – typical of a 'tropical oceanic' environment. Air movement was supplied through multiple apertures in one wall, and a rate of 5 m.p.h. was maintained daily for four to six hours. The air was conditioned outside the room, and a complete change of air was effected approximately every five minutes. The careful control of temperature and humidity provided a constant, standard environmental stress, the same for each subject and the same for each experimental period.

The fresh water provided was the emergency canned ration, standard in emergency kits in the R.C.A.F. and R.C.N. One can of fresh water daily was allotted to each man, and though 16 oz. was the amount each can was supposed to contain, actually by measurement each contained 450 cc. $\pm$ 2 cc, The sea water was obtained off the coast of New Brunswick and on analysis showed a chlorinity of 18.0 gm. per milliter (2.95% sodium chloride, or 507.0 m.e. per liter of chloride ion).

The food consumed was the R.C.A.F. dinghy ration Type C, comprising four 1-oz. chocolate bars, two 2-oz. packages of biscuit, lemon-flavored boiled candies, sugar cubes, and two packages of chewing gum. The caloric content of this ration is 1088 cal. and because three-quarters of the ration was consumed per day, the daily caloric intake of each subject was 816 cal. The sodium chloride content of the ration was found to be 1.88 gm. per package, so that the daily intake of sodium chloride from food was approximately 1.4 gm.

Clothing consisted of shorts, cotton shirt, and low canvas shoes or slippers for all subjects.

Methods

The experimental design was arranged to show differences in weight loss between subjects who received only the basic food and fresh water ration, and those who, in addition, received sea water. Any significant difference in the loss of weight of the two groups could then be considered to be due to differences in water storage or loss. Each subject remained in the chamber for seven days and nights continuously.

Four subjects at a time were studied and a total of eight subjects were employed, there being two experiments. *Two subjects in each experiment*

received 16 oz. of fresh water per day (plus food rations) and the other two received 16 oz. of fresh water and 10 oz. of sea water per day (plus food rations). Selection into 'sea water' and 'fresh water' (or 'control') groups was made, by tossing a coin, after entry into the chamber. Nude weights of all subjects were taken on hospital balance scales and were recorded to the nearest $\frac{1}{8}$ lb. (2 oz.) every two hours during waking hours. All urine voided was measured volumetrically, tested for reaction, blood (first morning sample only), albumin, ketone bodies, and specific gravity.

The subjects were allowed to take their water rations in the manner they found most suitable to their taste, the only provision being that the water was to be taken in divided doses during the day. The most commonly used method was to drink the sea water while eating the rations, and to finish off the meal with a drink of fresh water.

On the morning the subjects entered the chamber they had had neither food nor water for at least nine hours. Nothing was taken by mouth for another 24 hr. so that when the test period began, on the morning of the following day, all subjects were in water debt to an approximately equal degree.

During the first experiment the freezing points of the first morning samples of urine were determined, for estimation of total solutes. Each urine sample was retained in a labelled bottle until all determinations had been completed. The chloride ion concentration of each sample was determined. For the sodium and potassium estimations aliquot samples of each urine specimen of each day were mixed, to make representative samples for each day for each subject.

Blood samples were obtained before 'breakfast' each morning from each subject, and specific gravity, hematocrit, chloride, nonprotein nitrogen, and sodium estimations made on each.* Blood potassium studies were also planned, but could not be completed.

Close attention was given to the prevention of boredom in the subjects. Cards, a radio, a generous supply of books and magazines, and almost daily motion picture showings in the chamber were provided. Each man was carefully impressed with the necessity for consuming all and no more than his allowance of rations and water, and for reporting any deviations, however small. Each man kept a diary in which he recorded all relevant data respecting himself and his fellow subjects.

Results

The results of two experiments, each involving four men for a test period of six days (48 test days) were used as material.

* *Blood and urine sodiums were estimated by the wet ash method; the sodium was precipitated with zinc uranylacetate reagent; the color developed on solution of the precipitate was read by means of an Evelyn photoelectric colorimeter.*

Urine potassiums. After ashing the sample, the potassium was precipitated with chloroplatinic acid. The precipitate was then washed free of excess platinic acid with absolute alcohol. The amount of iodine set free by the addition of potassium iodide was then read with the photoelectric colorimeter.

Body Weight

It will be seen from Table I that the relative gain in body weight to the sea water group by comparison with the fresh water group falls within the generally accepted limits of statistical significance (P = 0.05).

TABLE I

ANALYSIS OF VARIANCE AND COVARIANCE OF INITIAL WEIGHT (X) AND FINAL WEIGHT (Y) FOR TEST PERIOD OF SIX DAYS

Control group (weight in pounds)			Sea water group (weight in pounds)		
Subject	Initial wt. (X)	Final wt. (Y)	Subject	Initial wt. (X)	Final wt. (Y)
M	175.25	165.25	D	129.25	123.00
McL	160.75	148.75	G	138.00	131.25
S	150.50	142.00	L	116.00	111.75
F	137.25	129.25	K	151.25	144.88

Sums of squares and products of deviation about the mean

	X^2	XY	Y^2*	D.f.*	Y^2 corrected	D.f.*	M.s.*	F*	P*
Within groups	1434.50	1339.78	1258.55	6	7.24	5	1.45	—	—
Between groups	947.14	825.35	687.25	1	10.74	1	10.74	7.42	0.05
Total	2431.64	2165.13	1945.80	7	17.98	6			

Mean corrected final weights

Control group	Sea water group	Difference	Significant difference for P = 0.05
135.89	138.13	2.24	2.19

* *D.f. = degrees of freedom, M.s. = mean square, F = variance ratio, P = probability.*

This table is a standard analysis of variance and covariance. The technique of, and theory behind this statistical method may be found in many standard statistical books such as Statistical Methods for Research Workers, Section 49.1 by R. A. Fisher (5), (Oliver & Boyd, 1938 and later). Essentially the method makes due correction for the lack of balance in the experiment caused by the unequal initial weights of the subjects. The corrected sum of squares for final weight corrected (Y^2 corrected) was computed from the dependence of final weight on initial weight in the "within groups" row, as the sums of squares and products in this row are independent of treatment. This correction involves the loss of one degree of freedom for estimating the significance of the difference of corrected final weights between groups, as this degree of freedom has been used to estimate the regression coefficient of final weight on initial weight. The probability (P) of the observed variance ratio (F) occurring by chance was obtained from Statistical Tables of Fisher and Yates (6).

Uneaten Rations

The total weight of rations not eaten by the control group was 15.0 oz. (subject McL., 13 oz.; M., 2 oz.; both in Expt. No. 1). (The sea water group ate all their rations.) If this weight is added to the final body weight

of the control group, the difference in the corrected weights for the control group would be about 2.0 lb. instead of 2.24 lb. (as calculated above), still close to the 5% limit of confidence. It can be argued that the whole weight of uneaten ration should not be added to the final body weights of the control subjects. An important fraction of the rations would be lost as carbon dioxide and water, and the protein-sparing action of the carbohydrates (resulting in less demand on water for removal of protein metabolites) would be offset largely or completely by the proteins and solutes present in the biscuits and chocolate.

Tables II and III incorporate the results of the various estimations performed on each subject, for each day of the test periods. Table II gives the data day by day for the first experiment, and Table III that for the second.

Water Balance

A study of the urine volumes of the two groups of subjects indicates that an appreciable quantity of the sea water ingested may have been retained in the body (Table IV).

If the sea water supplement had been excreted quantitatively the daily urine volume per man in the group receiving it might have been 702.9 cc. (295.7 cc. sea water + mean daily urine volume per man of control group), whereas it was actually 558.6 cc. (mean daily urine volume per man of sea water group), a presumptive gain of 145.3 cc. from each 295.7 cc. of sea water drunk. Evidence that this gain of water was beneficial is supported by the opinions expressed by the subjects themselves. (See below.)

Chloride Balance

There was no significant change in blood chloride levels in any subject during the tests, as determined from the blood samples taken each morning. There was a chloride debt in all controls (see Table V), the total intake of chloride ion for both test periods being 19.17 gm. and the total output 33.15 gm., a deficit of 13.98 gm. This deficit was further aggravated by loss of chloride through sweat, and in unacclimatized men in the environmental conditions set by the test, the estimated sweat chloride concentration would likely be at least 0.1%, accounting for at least several grams more. (No measurements of sweat chlorides were made.)

Though the loss of chloride through sweat operated to the disadvantage of the control group, it was advantageous to the sea water group by assisting the kidneys in ridding the body of the excess chloride ingested. As a group, the sea water subjects stored chloride during the test period, though during the final two days of the first experiment, Subjects D and G were actually excreting chloride in the urine faster than they were taking it in.

The rate of excretion of chloride ions by the control group of subjects fell to a rate of slightly more than 30 m.e. per day by the second day of the test period, where it remained for the remaining four days. The sea water group

TABLE II

LABORATORY DATA: FIRST EXPERIMENT

Subjects	Urine							Whole blood					
	Day of expt.	Daily vol., cc.	Sp. grav., 1st a.m. sample	Total solutes, 1st a.m. sample, mgm./100 cc.	Daily Cl ion, m. e.	Daily Na, m. e.	Daily K, m. e.	Cl ion, m. e. per liter	Na, m. e. per liter	Hematocrit	Hbg., %	Sp. grav.	NPN, mgm. %
Controls													
M	1	520	1025	5947	85.1	69.1	44.8	85.5	79.3	51.5	95.0	1.064	40
	2	410	1026	6351	43.0	29.3	40.6	84.9	79.3	52.0	97.0	1.064	38
	3	360	1025	5924	31.4	26.6	40.4	82.9	80.2	54.0	103.0	1.066	42
	4	380	1027	5884	29.3	30.1	44.7	96.1	77.6	52.5	101.0	1.067	45
	5	395	1028	6107	29.7	30.7	76.1	91.1	97.35	53.0	103.0	1.067	45
	6	355	1029	6403	33.4	34.6	47.9	84.2	82.0	51.5	101.7	1.066	47
	Total	2420			251.9	220.4	254.5						
McL	1	575	1023	6026	75.8	53.4	49.2	88.1	82.0	51.0	92.5	1.063	38
	2	345	1027	6053	39.1	27.8	39.3	99.3	82.0	51.0	95.0	1.062	37
	3	375	1029	6763	42.0	37.6	44.5	95.1	90.8	50.0	95.0	1.062	50
	4	360	1029	7337	43.0	40.3	42.3	87.5	82.0	50.0	93.8	1.063	49
	5	325	1027	6407	32.5	29.0	40.8	87.5	82.9	50.5	97.5	1.064	49
	6	500	1028	6683	34.6	45.1	52.1	82.8	81.1	51.2	97.0	1.064	45
	Total	2480			267.0	233.2	268.2						
'Sea water' subjects													
D	1	475	1021	5400	70.0	70.1	44.1	91.8	80.25	52.0	90.5	1.062	42
	2	720	1025	5652	161.5	106.5	65.4	86.9	82.9	49.5	90.5	1.062	33
	3	700	1022	5013	176.1	136.7	55.2	93.9	86.4	49.0	90.0	1.060	28
	4	565	1023	4798	152.0	127.4	44.1	87.6	76.7	49.0	91.3	1.061	37
	5	690	1025	5450	184.0	176.3	41.0	91.0	87.35	47.0	90.0	1.059	31
	6	720	1027	4966	184.1	168.0	46.2	87.6	82.0	51.0	97.0	1.062	—
	Total	3870			927.7	785.0	296.0						
G	1	514	1022	5388	70.9	42.1	58.0	87.0	79.3	52.5	97.5	1.063	45
	2	590	1022	5100	104.7	73.4	64.8	91.0	—	50.0	95.0	1.062	38
	3	630	1025	4698	164.5	136.7	59.9	96.0	81.7	51.0	98.3	1.064	38
	4	620	1023	5638	167.0	147.2	53.2	87.0	82.9	49.7	95.0	1.063	60
	5	630	1027	5042	175.8	158.3	47.1	87.5	82.0	51.5	99.0	1.063	45
	6	720	1030	5603	194.3	176.5	53.2	87.0	82.0	50.5	97.5	1.063	45
	Total	3704			877.2	734.2	336.2						

TABLE III

LABORATORY DATA: SECOND EXPERIMENT

Subjects	Urine						Whole blood					
	Day of expt.	Daily vol., cc.	Sp. grav., 1st a.m. sample	Daily Cl ion, m. e.	Daily Na, m. e.	Daily K, m. e.	Cl ion, m. e. per liter	Na, m. e. per liter	Hematocrit	Hbg., %	Sp. grav.	NPN, mgm. %
Controls												
F	1	580	1.023	55.9	31.3	58.0	87.0	72.2	56.5	99.8	1.065	40
	2	450	1.023	33.5	18.4	46.4	87.5	77.5	55.0	98.3	1.063	42
	3	385	1.023	21.8	24.6	33.7	87.5	75.8	56.0	100.2	1.064	40
	4	355	1.026	20.9	23.2	36.8	90.2	75.8	56.3	101.7	1.065	35
	5	330	1.027	35.9	16.7	37.1	84.9	71.5	57.0	102.5	1.065	42
	6	365	1.026	18.8	23.4	47.9	87.0	82.0	56.0	101.0	1.065	40
	Total	2465		186.8	137.6	259.9						
S	1	494	1.032	52.2	57.2	39.1	87.5	80.2	53.0	97.5	1.064	40
	2	427	1.026	49.5	28.3	30.2	87.5	82.9	51.0	95.8	1.063	40
	3	380	1.025	34.5	23.8	30.2	94.9	82.9	52.5	97.0	1.064	42
	4	387	1.024	33.6	31.0	30.9	89.6	78.4	53.0	100.2	1.066	35
	5	345	1.026	27.1	33.6	31.0	87.0	78.4	53.2	99.8	1.065	45
	6	375	1.029	28.8	31.9	36.6	86.3	85.5	51.5	97.5	1.064	42
	Total	2408		225.7	205.8	198.0						
'Sea water' subjects												
K	1	442	1.024	114.1	74.2	60.6	85.5	96.9	49.5	88.1	1.060	50
	2	378	1.025	85.9	62.4	27.1	85.0	85.5	48.5	88.1	1.060	31
	3	366	1.024	92.9	71.4	33.7	87.5	86.5	50.0	91.3	1.060	31
	4	465	1.024	130.5	117.1	35.8	89.6	86.5	48.5	90.0	1.060	35
	5	535	1.026	147.9	134.9	50.2	86.2	85.5	48.0	88.1	1.058	37
	6	482	1.024	133.0	121.3	38.8	89.0	91.0	47.5	88.1	1.059	38
	Total	2668		704.3	581.3	246.2						
L	1	600	1.019	104.1	75.3	51.4	87.5	82.0	50.0	91.3	1.061	40
	2	565	1.026	144.2	97.6	61.2	87.5	84.6	48.0	89.3	1.059	42
	3	495	1.028	130.5	106.6	45.9	90.5	85.5	49.0	88.8	1.059	37
	4	535	1.024	145.0	128.2	40.7	94.5	82.0	49.2	92.5	1.061	33
	5	510	1.024	150.1	114.0	41.8	93.1	81.1	50.5	93.8	1.061	42
	6	460	1.029	132.2	121.8	30.4	95.2	85.5	50.0	93.0	1.062	45
	Total	3165		806.1	643.5	271.4						

TABLE IV

URINE VOLUMES

	Daily urine volumes (cc.) : totals							Mean daily urine volume per subject during test period	S.d.
	'Dehydration day'	1	2	3	4	5	6		
Control group	2810	2169	1632	1500	1482	1395	1595	407.2	± 71.0
'Sea water' group	2732	2031	2253	2191	2185	2365	2382	558.6	± 106.5

TABLE V

CHLORIDE BALANCE

Total m. e. of chloride ion for each group for all six days of test period

	Intake		Output (excluding loss in sweat and feces)
Control group	Rations	539.87	933.7
'Sea water' group	Rations	539.87	
	Sea water	3619.6	
		4159.47	3353.63

excreted an average of approximately 160 m. e. chloride ion per day during the last two days of the test period, slightly less than the amount ingested daily (Fig. 1). The maximum chloride ion concentration during the tests in any urine sample was 320.1 m.e. per liter representing approximately 1.9% of chloride salt.

There were no symptoms recorded by any subject or signs noted on physical examination that could with reasonable certainty be attributed to salt deficiency or salt storage.

Urine Sodium and Potassium

The sodium excretion in both groups of subjects closely paralleled the excretion of chlorides. The expected high urine sodium values in the group receiving sea water are clearly evident (Fig. 2). The excretion of sodium increased daily until the fifth day where it levelled off at a mean value of 146 m.e. The control group appeared to reach a steady rate of excretion around 30 m.e., closely tallying with the corresponding chloride excretion.

The excretion of *potassium* during the test period was greater by the group receiving sea water (17.2% more than the control group). However, the rate of excretion towards the end of the test period, as shown in the graph

(Fig. 3) was approximately the same in each, with the difference favoring the sea water group. From this, it appears that the sea water group lost more intracellular water during the first half of the test period, but in comparison with the control group they lost water from their cells at no greater rate during the final two days.

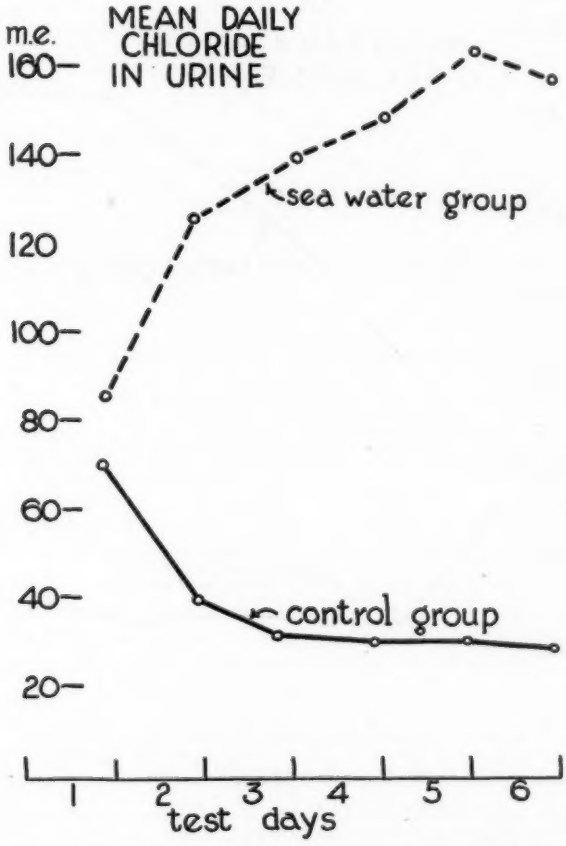

FIG. 1. *Mean daily chloride in urine. Each point represents the mean chlorine ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of chloride ion in urine. The abscissa = test days.*

Blood Sodiums

The blood sodium levels remained within normal limits throughout both test periods, and there was no significant difference between the values for the control and sea water groups. (N.B.—The blood sodium and chloride values given in Tables II and III refer to *whole* blood determinations.)

Examination of Nitrogenous Metabolites

There was no evidence of retention of nitrogenous metabolites in either group of subjects, as revealed by the blood nonprotein nitrogen levels. As shown by the graphs, it is clear that in general, the subjects receiving sea water excreted the nitrogenous substances fully as satisfactorily as did the

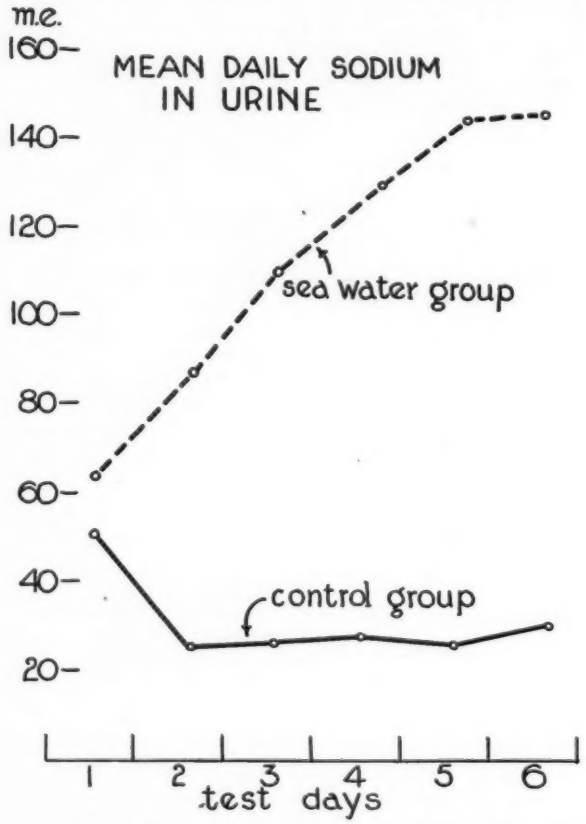

FIG. 2. *Mean daily sodium in urine. Each point represents the mean sodium ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of sodium ion in urine. The abscissa = test days.*

control group. The highest reading (60 mgm. %*) was obtained on the morning of the fourth day of the first test period from a sea water subject (G). On that day he reported "0850: didn't sleep so well last night. My mouth is like the bottom of a bird cage. I am so—thirsty this morning." "1830 urine 160 cc. Felt—awful between 3 and 6 o'clock, but feel better now. Slightly weak and lack the pep that I had on Tuesday" (the day of dehydration preliminary to the test period). Examination at 5 p.m. showed nothing

**Mgm. % = mgm. by weight of ion per 100 cc. of solvent.*

unusual. The tongue was clean and the subject was alert and seemed in good spirits. All examinations, physical and laboratory, were noncontributory. The following and subsequent days the blood NPN estimation for this subject was 45 mgm. %.

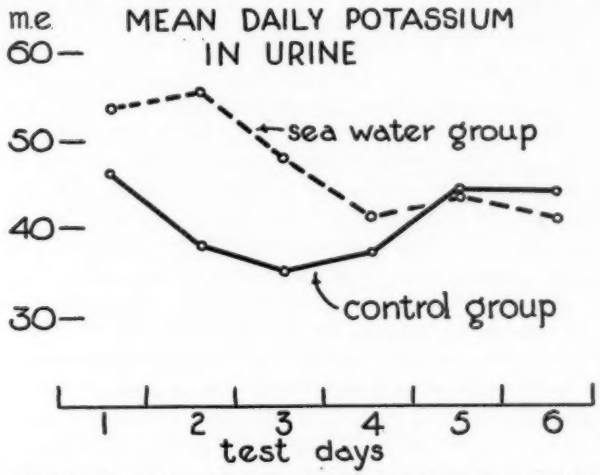

FIG. 3. *Mean daily potassium in urine. Each point represents the mean potassium ion excretion of all four subjects in each group, for one day. The ordinate = m. e. of potassium ion in urine. The abscissa = test days.*

Other Urine Examinations

In all subjects no evidence of renal damage appeared, as shown by tests for albumin and blood of all samples. The urine was generally acid in all subjects, though several men occasionally passed an alkaline sample. Ketone bodies were noted in three out of four subjects in the second experiment, reached a peak on the second day in the chamber (first day of the test period), and disappeared during the third day, never to reappear.

The urine specific gravity varied little between the two groups. During the first experiment, the average specific gravity of the urine of the sea water subjects was almost four points lower than that of the control group. The difference was in the same direction in the second experiment, but was small.

The highest specific gravity recorded was 1032 (fresh water subject S).

The percentage concentrations of total solutes in the urines of the fresh water subjects during the first experiment were approximately 1 gm. % higher than in the urines of those receiving the sea water supplement. However, the actual weight of solutes excreted by the sea water group was higher owing to the larger volume of urine put out.

Blood Specific Gravity, Hemoglobin, Hematocrit

The results of these three tests closely paralleled each other. The values, as shown in Tables II and III, rose appreciably during the test period and

fell to normal rapidly when the restrictions on diet and fluid intake were removed. The sea water supplement apparently was instrumental in preventing much rise in blood concentration and although, for instance, there was a rise of 3 to 5 in the hemoglobin percentages, the corresponding rise for the subjects on fresh water alone was 4 to 8 above the figures obtained before entry into the test room.

Consumption of Rations

At first there was little difficulty in consuming the food rations, but during the final two days of the experiment, the difficulty was extreme for all control subjects. Two of the control subjects were unable to eat their rations completely and for subject McL. this amounted to a total of 13 oz. for the last two days, and to 2.3 oz. for subject M. All items in the rations were rejected (chocolate and biscuits, as well as sugar and candy).*

The complete consumption of all sea water rations was an easy task by comparison with the difficulty of eating the food. One subject (K, on sea water) was a notable exception. He was not conscious of thirst during the test period. Opinion was divided as to whether sea water relieved thirst, but none said it made thirst more pronounced. Nausea was less prominent with the sea water subjects, but this, it is believed, was mainly because of the greater difficulty the control group had in eating their rations. Nausea was an invariable accompaniment of eating rations for the control subjects during the last two days of the test period.

Physical Condition of the Subjects

As no performance tests were included in the test program, almost all the evidence of the physical condition of the subject is necessarily qualitative in type.

There was no major change in the *appearance* of any of the subjects during the test period, and none appreciable until the second test day. By the fourth day, all subjects were showing evidences of weakness, and their faces were 'pinched' and pale. Posture and gait indicated loss of muscular tone and movements generally were rather slowly performed. These changes were thought to be less evident in the subjects receiving sea water. During the last two days of the test period, those who received sea water seemed more alert and less depressed.

The *saliva* became almost solid in consistency towards the end of the test, and had a distinctly foul taste and odor.

The blood pressure and pulse rates of subjects in the reclining and sitting postures were recorded at the same hour daily. Nothing significant of any abnormality was found.

Marked *constipation* was present in all subjects. Feces usually consisted of not more than three or four scybalous masses, 1 in. in diameter or less.

** The great effort to consume the rations is exemplified by one subject. After choking down his 'meal', he repeatedly regurgitated it into his mouth. It was only by keeping his hand over his mouth that he prevented the loss of the rations.*

Most of the subjects had no more than two bowel movements during the test period.

Subjective Data

Most complaints were of *thirst.* Thirst was marked in degree in two control subjects, both of whom were so obsessed with it that they got out of bed during the fifth night and drank 2 oz. of their water rations for the following day.

Insomnia was troublesome for all subjects during the last three nights, and often appeared to be due to a gnawing pain in the abdomen (usually epigastric).

A sense of *weakness* was first noted by most subjects on the third test day. On the following day and subsequently, noticeable tiredness and lack of ambition was recorded by three of the control subjects and by one sea water subject. In the first experiment the control subjects were convinced that they had appreciably less stamina than those who were drinking sea water. This view was expressed by one control subject as follows: "G and D (sea water subjects) both mentioned that they could last another week if they had to . . . . . I know, for one, that I would be a stretcher case if I had to stick it out another week and I think I can safely say that M (other control subject) feels the same way." (M confirmed this in his diary.)

Boredom was a universal complaint during the last two test days, but never became an important problem. There were no symptoms or signs of mental aberrance at any time during or following the test.

Recovery Period

The subjects reported for observation several times during the day of their release, the following morning, and one week later. No untoward symptoms or signs appeared in any subject. All showed a surprisingly rapid recovery. Within six hours after the first meal there was a noticeable return of normal skin color and alertness, together with a surge of buoyant cheerfulness. No difference was detectable between the two groups of subjects in the length or character of their respective recovery periods.

Slight epigastric discomfort, consisting mainly of a feeling of distension and occasionally of burning was noted during the first day after leaving the chamber. (This symptom probably was due to mechanical dilatation of the stomach by unaccustomed volumes of food and fluid.)

Value of Drinking Sea Water; Opinions Expressed by the Subjects

All subjects felt that the extra supplement of sea water resulted in less loss of stamina and that it facilitated the chewing and swallowing of the rations. All thought that if they were in a dinghy at sea and had only a small supply of fresh water, they would drink sea water with the fresh water rations.

Discussion

An experiment of this scope and character can only point the way to a more precise and more practical study of the problem. The influence of sea-sickness, exposure to the weather, and mental stress may severally or in concert

render the use of sea water in any quantity inadvisable. From this experiment, it is evident that under the conditions of the test, some benefit without any important disadvantages was conferred by the addition of sea water to the fresh water rations. Further studies are desirable to improve the precision and predictability of the results, and it is clear that no instructions respecting the use of sea water should be formulated until actual trials in a dinghy at sea have been completed. In preparing for such trials, it would seem advisable to replace the sodium salts in the food rations with potassium salts, and thereby permit the castaway to make better use of the sea water supplement to his fresh water ration.

It appears to the authors that if men can be trusted to follow instructions to avoid sea water, they could also be trusted to follow instructions to utilize it to their best advantage.

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